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Cell-based ELISA

Catalog number: EKC2479

For the quantitation of **Human, Mouse, Rat NF-kappaB p65 (Phospho-Thr435)** concentrations in

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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NF-kappaB p65 (Phospho-Thr435) Colorimetric Cell-Based ELISA Kit

Catalog Number:EKC2479

Assay Principle

The Colorimetric Cell-Based ELISA Kit allows for the detection of various target proteins and the effects that certain stimulation conditions have on target protein expression in different cell lines. Qualitative determination of target protein concentration is achieved by an indirect ELISA format. In essence, the target protein is captured by target-specific primary (1°) antibodies while the HRP-conjugated secondary (2°) antibodies bind the Fc region of the 1° antibody. Through this binding, the HRP enzyme conjugated to the 2° antibody can catalyze a colorimetric reaction upon substrate addition. Due to the qualitative nature of the Cell-Based ELISA, multiple normalization methods are described: 1) a monoclonal antibody specific for human GAPDH is included to serve as an internal positive control in normalizing the target absorbance values. 2) Following the colorimetric measurement of HRP activity via substrate addition, the crystal violet whole-cell staining method is used to determine cell density. After staining, the results can be analyzed by normalizing the absorbance values to cell amounts, by which the plating difference can be adjusted. 3) If a phosphorylated target is being detected, an antibody against the nonphosphorylated counterpart will be provided for normalization purposes. The absorbance values obtained for the non-phosphorylated target can be used to normalize the absorbance values for the phosphorylated target. used to normalize the absorbance values for the phosphorylated target.

Overview

Product Name	NF-kappaB p65 (Phospho-Thr435) Colorimetric Cell-Based ELISA Kit
Reactive Species	Human, Mouse, Rat
Size	1 kit, containing two 96-well plates and all necessary reagents
Description	The NF-kappaB p65 (Phospho-Thr435) Cell-Based ELISA Kit is a convenient, lysate-free, high throughput and sensitive assay kit that can monitor NF-kappaB p65 (Phospho-Thr435) protein expression profile in cells. The kit can be used for measuring the relative amounts of NF-kappaB p65 (Phospho-Thr435) in cultured cells as well as screening for the effects that various treatments, inhibitors (ie. siRNA or chemicals), or activators have on NF-kappaB p65 (Phospho-Thr435).
Sensitivity	
Detection Range	> 5000 cells/well
Storage Instructions	Store at 4°C for up to 6 months.
Uniprot ID	Q04206

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Kit Components/Materials Provided

Reagent	Quantity	Container
96-Well Cell Culture Clear-Bottom Microplate	2 Plates	-
10x TBS	30 ml (10x)	Clear
Quenching Buffer	24 ml (1x)	Clear
Blocking Buffer	50 ml (1x)	Clear
15x Wash Buffer	60 ml (15x)	Clear
100x Anti-NF-kappaB p65 Phospho-Thr435 Antibody (Rabbit Polyclonal)	60 µl (100x)	Red
100x Anti-NF-kappaB p65 Antibody (Rabbit Polyclonal)	60 µl (100x)	Purple
100x Anti-GAPDH Antibody (Mouse Monoclonal)	60 µl (100x)	Green
HRP-Conjugated Anti-Rabbit IgG Antibody	12 ml (1x)	Plastic
HRP-Conjugated Anti-Mouse IgG Antibody	12 ml (1x)	Plastic
Primary Antibody Diluent	12 ml (1x)	Clear
Ready-to-Use Substrate	12 ml (1x)	Brown
Stop Solution	12 ml (1x)	Clear
Crystal Violet Solution	12 ml (1x)	Plastic
SDS Solution	24 ml (1x)	Clear
Adhesive Plate Seals	2 Seals	-

Required Materials That Are Not Supplied

- Microplate reader able to measure absorbance at 450 nm and/or 595 nm for Crystal Violet Cell Staining (Optional)
- Micropipettes with capability of measuring volumes ranging from 1 ul to 1 ml
- 37% formaldehyde (Sigma Cat# F-8775) or formaldehyde from other sources
- Deionized or sterile water
- Squirt bottle, manifold dispenser, multichannel pipette reservoir or automated microplate washer
- Graph paper or computer software capable of generating or displaying logarithmic functions
- Absorbent papers or vacuum aspirator
- Test tubes or microfuge tubes capable of storing 1 ml
- Orbital shaker
- Poly-L-Lysine (Sigma Cat# P4832 for suspension cells)

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Preparation Before The Experiment

Item	Preparation
1x TBS	1x TBS is used to wash cells seeded on the plate. 1x TBS can be prepared by adding 1 volume of 10x TBS provided in the kit to 9 volumes of ddH2O.
Fixing Solution	This solution is NOT provided. Fixing Solution is used to fix cells after cell culture. It is prepared by adding formaldehyde to 1x PBS (not included) with light mixing. The 4% formaldehyde is used for adherent cells and 8% formaldehyde is used for suspension cells and loosely attached cells. 37% formaldehyde can be purchased from Sigma Cat# F8775.
Quenching Buffer	This solution is provided as ready-to-use. Quenching Buffer is used to inactivate the endogenous peroxidase activity of the seeded cells.
Blocking Buffer	This solution is provided as ready-to-use. Blocking Buffer is used to block additional binding sites in each well.
1x Wash Buffer	This buffer is provided as a 15x solution. 1x Wash Buffer can be prepared by adding 1 volume of 15x Wash Buffer provided in the kit to 14 volumes of ddH2O.
100x Anti-NF-kappaB p65 (Phospho-specific) Antibody	This antibody is a rabbit polyclonal antibody. This antibody was tested to be specific for the phosphorylated NF-kappaB p65 protein. The supplied antibody is a 100x solution. Make 1:100 dilutions in Primary Antibody Diluent prior to use. The diluted primary antibody can be stored at 4°C for up to two weeks.
100x Anti-NF-kappaB p65 Antibody	This antibody is a rabbit polyclonal antibody. This antibody was tested to be specific for the NF-kappaB p65 protein. The supplied antibody is a 100x solution. Make 1:100 dilutions in Primary Antibody Diluent prior to use. The diluted primary antibody can be stored at 4°C for up to two weeks.
100x Anti-GAPDH Antibody	This antibody is a mouse monoclonal antibody. This antibody was tested to be specific for GAPDH. The supplied antibody is a 100x solution. Make 1:100 dilutions in Primary Antibody Diluent prior to use. The diluted primary antibody can be stored at 4°C for up to two weeks.
HRP-Conjugated Anti-Rabbit IgG Antibody	This solution is provided as ready-to-use. HRP-Conjugated Anti-Rabbit IgG Antibody is used as the secondary antibody to detect the target-bound, primary rabbit antibodies.
HRP-Conjugated Anti-Mouse IgG Antibody	This solution is provided as ready-to-use. HRP-Conjugated Anti-Mouse IgG Antibody is used as the secondary antibody to detect the target-bound, primary mouse antibodies.
Primary Antibody Diluent	This solution is provided as ready-to-use. Use this solution to dilute the provided antibodies.
Ready-to-Use Substrate	This solution is provided as ready-to-use. Ready-to-Use Substrate must be warmed to room temperature before use. Keep away from light as this solution is light-sensitive.
Stop Solution	This solution is provided as ready-to-use. Stop Solution must be handled with caution as it contains 2 N Sulfuric Acid (H2SO4) and is corrosive. Wear eye protection and gloves when handling.

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Crystal Violet Solution	This solution is provided as ready-to-use. Crystal Violet is an intense stain used to stain cell nuclei. Avoid contact with skin and clothing.
SDS Solution	This solution is provided as ready-to-use. SDS is used to solubilize the Crystal Violet in preparation for cell staining. Store this solution at room temperature or warm up to room temperature if stored at 4°C.
Adhesive Plate Sealers	Provided for long term storage of plate if necessary.

Health And Safety Precautions

- 1. Reagents provided in this kit may be harmful if ingested, inhaled orabsorbed through the skin.
- 2. Fixing Solution contains formaldehyde. Formaldehyde is known to bea highly toxic reagent. Personal protection is strongly recommendedwhile working with this chemical.
- 3. Stop Solution contains 2 N Sulfuric Acid (H2SO4) and is an extremelycorrosive agent. Please wear proper eye, hand and face protectionwhen handling this material. When the experiment is finished, be sureto rinse the plate with copious amounts of running water to dilute theStop Solution prior to disposing the plate or strips.
- 4. Crystal Violet is an intense stain reagent. Avoid contact stain andclothing.

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Preparation Before The Experiment

Cell Line	The cell line must express the target protein. This protocol can be used directly for adherent cells. For suspension cells and loosely attached cells, two steps are required: 1) Coat the plates with 100 µl of 10 µg/ml Poly-L-Lysine (Sigma Cat# P4832, not included) to each well of the 96-well plate for 30 minutes at 37°C before proceeding to Step 1 of Assay Protocol (on page 13). Use 8% formaldehyde to fix the cells on Step 5 of Assay Protocol.
Cell Number and Sensitivity	The number of cells plated onto the 96-well plates depends on the expression level of NF-kappaB p65 protein in the cells, cell size, treatment conditions and incubation time. The cells used for testing should be around 75-90% confluent. Typically for cells, seed 30,000 cells per well overnight for treatment the following day. The NF-kappaB p65 Colorimetric Cell-Based ELISA Kit can detect NF-kappaB p65 expression in as few as 5,000 cells.
Cell Treatment	The cells can be treated with inhibitors, activators, stimulators (ie. chemicals, proteins/peptides) or a combination of the substances listed above. The cells can be treated with UV and serum starvation to meet the needs of the end-user.
Positive and Negative Controls	Positive control: GAPDH is the internal positive control for the assay. Mouse Anti-GAPDH Antibody (included) detects GAPDH and GAPDH's O.D. values are used to normalize the O.D. values of the target protein. Negative control: independent wells of cells treated with only with HRP-Conjugated Anti-IgG Antibodies, and without primary antibodies. Both positive and negative controls should be performed in the same plate with the NF-kappaB p65 target experiments.
Accuracy and Precision	Each condition should be performed in duplicate or in triplicate.

Assay Protocol

1) Seed 200 µl of 20,000 adherent cells in culture medium in each well of a 96-well plate. The plates included in the kit are sterile and treated for cell culture. For suspension cells and loosely attached cells, coat the plates with 100 µl of 10 µg/ml Poly-L-Lysine (not included) to each well of a 96-well plate for 30 minutes at 37°C prior to adding cells.

2) Incubate the cells for overnight at 37°C, 5% CO2.

3) Treat the cells as desired.

4) Remove the cell culture medium and rinse with 200 µl of 1x TBS, twice.

5) Fix the cells by incubating with 100 µl of Fixing Solution for 20 minutes at room temperature. The 4% formaldehyde is used for adherent cells and 8% formaldehyde is used for suspension cells and loosely attached cells. During the incubation, the plates should be sealed with Parafilm. Note: Fixing Solution is volatile. Wear appropriate personal protection equipment (mask, gloves and glasses) when using this chemical.

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6) Remove the Fixing Solution and wash the plate 3 times with 200 µl 1x Wash Buffer for five minutes each time with gentle shaking on the orbital shaker. The plate can be stored at 4°C for a week. Note: For all wash steps, tap the plate gently on absorbent papers to remove the solution completely.

7) Add 100 µl Quenching Buffer and incubate for 20 minutes at room temperature.

8) Wash the plate 3 times with 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

9) Add 200 µl of Blocking Buffer and incubate for 1 hour at room temperature.

10) Wash 3 times with 200 µl of 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

11) Add 50 µl of 1x primary antibodies (Anti-NF-kappaB p65 (Phospho-specific) Antibody, Anti-NF-kappaB p65 Antibody and/or Anti-GAPDH Antibody) to the corresponding wells, cover with Parafilm and incubate for 16 hours (overnight) at 4°C. If the target expression is known to be high, incubate for 2 hours at room temperature with gentle shaking on the shaker.

12) Wash 3 times with 200 µl of 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

13) Add 50 µl of 1x secondary antibodies (HRP-Conjugated AntiRabbit IgG Antibody and/or HRP-Conjugated Anti-Mouse IgG Antibody) to corresponding wells and incubate for 1.5 hours at room temperature with gentle shaking on the shaker. Note: Add HRP-Conjugated Anti-Rabbit IgG Antibody to the wells incubated with Anti-NF-kappaB p65 and Anti-Phosphorylated NF-kappaB p65 (rabbit, polyclonal), and add HRP-Conjugated Anti-Mouse IgG Antibody to the wells incubated with Anti-GAPDH Antibody (mouse, monoclonal).

14) Wash 3 times with 200 µl of 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

15) Add 50 µl of Ready-to-Use Substrate to each well and incubate for 30 minutes at room temperature in the dark with gentle shaking on the shaker. Note: Ready-to-Use Substrate is a light-sensitive reagent. Keep away from light.

16) Add 50 µl of Stop Solution to each well and read OD at 450 nm immediately using the microplate reader. | NF-kappaB p65 | 15 Optional: Crystal Violet Cell Staining Crystal Violet binds to cell nuclei and gives absorbance readings proportional to cell counts at 595 nm.

17) After finishing reading the absorbance at 450 nm, wash the plate twice with 200 µl of Wash Buffer and twice with 200 µl of 1x TBS for 5 minutes each. Tap the plates on paper towel to remove the excess liquid. Let plate air dry for 5 minutes at room temperature.

18) Add 50 µl of Crystal Violet Solution to each well, incubate for 30 minutes at room temperature on the shaker. Note: Crystal Violet is an intense stain. Avoid contact with skin and clothing.

19) Tip off Crystal Violet solution into beaker. Wash plate by dipping into bucket of water in the sink with the water continuing to run. Carefully rinse the wells in distilled water until no more color comes off the wells. Allow the plate to dry for 30 minutes.

20) Add 100 µl of SDS Solution into each well and incubate on the shaker at room temperature for 1 hour.

21) Read absorbance at 595 nm with microplate reader. If absorbance is too high, the solubilized Crystal Violet Solution can be diluted 10 times with H2O on a separate 96-well plate.

Data Normalization

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Anti-NF-kappaB p65 Antibody Normalization:

The OD values obtained for the phosphorylated target protein can be normalized using the OD values obtained for the non-phosphorylated target protein via the proportion, OD450 (Anti-NF-kappaB p65 (Phosphorylated) Antibody)/OD450 (Anti-NF-kappaB p65 Antibody).

GAPDH Normalization:

The OD450 values obtained for the target protein can be normalized using the OD450 values obtained for GAPDH.

Crystal Violet Staining Normalization

The measured OD450 readings can be normalized using the OD595 values via the proportion, OD450/OD595.

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